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Regulation of IL-1-induced selective IL-6 release from human mast cells and inhibition by quercetin

[1,2,5]Kristiana Kandere-Grzybowska, [2]Duraisamy Kempuraj, [1,2]Jing Cao, [3]Curtis L. Cetrulo & *,[1,2,4]Theoharis C. Theoharides

[1]Department of Biochemistry, Tufts University School of Medicine and Tufts – New England Medical Center, Harrison avenue, Boston, MA, U.S.A.; [2]Department of Pharmacology & Experimental Therapeutics, Tufts University School of Medicine and Tufts – New England Medical Center, Harrison avenue, Boston, MA, U.S.A.; [3]Department of Obstetrics and Gynecology, Tufts University School of Medicine and Tufts – New England Medical Center, Harrison avenue, Boston, MA, U.S.A. and [4]Internal Medicine, Tufts University School of Medicine and Tufts – New England Medical Center, Harrison avenue, Boston, MA, U.S.A.

1 Mast cells are involved in allergic reactions, but also in innate immunity and inflammation. Crosslinkage of mast cell Fc immunoglobulin E receptors (FcεRI) by multivalent antigen triggers secretion of granule-stored mediators, as well as *de novo* synthesis of cytokines, including interleukin (IL)-6.

2 We showed recently that the proinflammatory cytokine IL-1 stimulates human leukemic mast cells (HMC-1) and human umbilical cord blood-derived cultured mast cells (hCBMCs) to release newly synthesized IL-6 without tryptase in the absence of degranulation.

3 Here, we investigated several signal-transduction pathways activated by IL-1 leading to IL-6 production by HMC-1 and hCBMCs.

4 We also investigated the effect of the flavonol quercetin that was recently shown to strongly inhibit IL-6 secretion in response to allergic stimulation from hCBMCs.

5 IL-1 stimulated p38, but did not activate extracellular signal-regulated kinase (ERK) or c-jun N-terminal kinase (JNK); it also did not activate protein kinase C (PKC) isozymes α, β, μ and ζ, except for PKC-θ, which was phosphorylated.

6 The p38 inhibitor SB203580 and the PKC inhibitors Calphostin C and Gö6976 completely inhibited IL-1-induced IL-6 production.

7 Quercetin 1–100 μM inhibited IL-1-induced IL-6 secretion, p38 and PKC-θ phosphorylation in a dose-dependent manner.

8 These results indicate that IL-1-stimulated IL-6 production from human mast cells is regulated by biochemical pathways distinct from IgE-induced degranulation and that quercetin can block both IL-6 secretion and two key signal transduction steps involved.
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Abbreviations: FcεRI, Fc immunoglobulin E receptors; hCBMCs, human umbilical cord blood-derived cultured mast cells; HMC-1, human leukemic mast cell line; IC_{50}, inhibitory concentration for 50%; IL, interleukin; MC, mast cell; NF-κB, nuclear factor kappa B; PKC, protein kinase C; SCF, stem cell factor

Introduction

Mast cells are necessary for the development of allergic reactions, but have been increasingly implicated in innate and acquired (Galli *et al*., 2005) immunity, as well as in inflammatory diseases (Theoharides & Cochrane, 2004). Crosslinkage of IgE receptors (Fc immunoglobulin E receptors, FcεRI) by multivalent antigen in mast cells results in secretion of granule-associated mediators and release of *de novo* synthesized cytokines, including interleukin (IL)-6 (Wedemeyer *et al*., 2000; Marone *et al*., 2002). FcεRI crosslinkage leads to a number of distinct signaling steps (Metzger *et al*., 2002) that involve calcium influx, as well as activation of mitogen-activated protein (MAP) kinases, in a serine/threonine protein kinase C (PKC)-dependent or -independent manner (Zhang *et al*., 1997; Kawakami *et al*., 1998; Siraganian, 2003). All isozymes of PKC have been implicated in IgE-induced release of preformed mediators (Ozawa *et al*., 1993a; Chang *et al*., 1997; Kimata *et al*., 1999), as well as in cytokine production (Razin *et al*., 1994; Chang *et al*., 1997); these include the classical calcium-sensitive isozymes α, β; the calcium-insensitive δ, ε, θ, as well as the atypical ζ isozyme (White & Metzger, 1988; Ozawa *et al*., 1993b; Liu *et al*., 2001). The calcium and phorbol ester-independent PKC-θ was shown to augment antigen-induced degranulation of rat basophil leukemia (RBL) cells (Liu *et al*., 2001). All three MAP kinase

*Author for correspondence at: Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston MA 02111, U.S.A.;
E-mail: theoharis.theoharides@tufts.edu
[5]Current address: Department of Cell and Molecular Biology, Northwestern University Medical School, 303 E Chicago Avenue, Ward 8-063, Chicago IL 60208-3008, U.S.A.

pathways p38, ERKs and JNKs have been implicated in regulation of IL-6 mRNA or protein production in response to FcεRI aggregation (Offermanns *et al*., 1994).

Activation of MAP kinases and PKC are typical signaling events induced by the proinflammatory cytokine interleukin-1 (IL-1) in several cell types (Dinarello, 1994). With few exceptions, IL-1 induces formation of diacylglycerol (DAG), ceramide or phosphatidic acid in the absence of phosphatidylinositol hydrolysis and, therefore, does not raise cytoplasmic calcium (Dinarello, 1994). The atypical PKC isozyme ζ is, therefore, most consistently activated by IL-1 in different cell types (Ganz *et al*., 1996; Rzymkiewicz *et al*., 1996; Esteve *et al*., 2002) and has been implicated in activation of ERKs (Berra *et al*., 1995).

We recently showed that IL-1 stimulates *selective* release of newly synthesized IL-6 through a calcium-independent process that does not involve degranulation (Kandere-Grzybowska *et al*., 2003). We also recently showed that certain flavonols, especially quercetin, could inhibit FcεRI-induced cytokine release from human umbilical cord blood-derived mast cells (hCBMCs) and phosphorylation of PKC-θ (Kempuraj *et al*., 2005). Here, we investigated the effect of IL-1 on phosphorylation of p38, ERK and JNK, as well as activation of PKC isozymes in human leukemic mast cells (HMC-1) and in hCBMCs. We also showed that pretreatment with quercetin inhibits IL-1-induced IL-6 release, as well as PKC-θ and p38 activation. We are not aware of any previous studies on activation of intracellular signaling pathways in human mast cells by IL-1 or its inhibition.

Methods

Human recombinant IL-1α, IL-4, IL-6 and TNF-α were purchased from Chemicon Inc. (Temecula, CA, U.S.A.). Recombinant human stem cell factor (rhSCF) was a gift from Amgen, Inc. (Thousand Oaks, CA, U.S.A.). Monoclonal mouse antibody to PKC-α was from Upstate Cell Signaling Solutions (Lake Placid, NY, U.S.A.) and to PKC-β from BD Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal rabbit antibodies to PKC-ζ and actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and phosphorylated (p) PKC isozymes p-PKC-μ, p-PKC-ζ and p-PKC-θ were from Cell Signaling Technologies (Beverly, MA, U.S.A.). Stock solutions of SB203580, an inhibitor of the p38 MAP kinase, and of the PKC inhibitors Calphostin C and Gö6976 (Table 1, all from Calbiochem, EMD Bioscience Inc., La Jolla, CA, U.S.A.) were prepared in DMSO and diluted so that the final DMSO concentration was <0.1%. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) was from Cell Signaling and goat anti-mouse HRP-conjugated antibody was from Upstate Signaling Solutions. Quercetin was obtained from Sigma (St Louis, MO, U.S.A.) and was diluted in DMSO prior to final dilutions in culture medium; the final DMSO concentration was <1%.

Mast cell culture

Human cord blood was obtained from normal deliveries in accordance with established institutional guidelines. hCBMCs were derived by the culture of $CD34^+$ progenitor cells as previously described (Kempuraj *et al*., 1999) with minor modifications. Briefly, mononuclear cells were isolated by pipetting heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical, Aurora, OH, U.S.A.). $CD34^+$ progenitor cells were isolated from mononuclear cells by selection of cells positive for the AC133 antigen ($CD133^+$/$CD34^+$) by magnetic cell sorting (Miltenyi Biotec, Auburn, CA, U.S.A.). For the first 4 weeks, $CD34^+$ cells were cultured in IMDM (GIBCO BRL, Long Island, NY, U.S.A.) supplemented with 0.55 μM 2-mercaptoethanol, 100 mg l^{-1} Insulin-Transferrin-Selenium supplement (ITS) from GIBCO, 0.1% bovine serum albumin (BSA; Sigma), 1% penicillin/streptomycin (GIBCO), 100 ng ml^{-1} SCF (Amgen) and 50 ng ml^{-1} IL-6 (Chemicon) at 37°C in 5% CO_2-balanced air. After 4 weeks of culture, BSA and ITS in the culture medium were substituted with 10% fetal bovine serum (FBS) (GIBCO). By 8 weeks, 95% of the cells in the culture were identified as mast cells by immunostaining for tryptase. HMC-1 cells were obtained from Dr J.H. Butterfield (Mayo Clinic, St Paul, MN, U.S.A.) and were cultured in IMDM supplemented with 10% FBS (Hyclone, Logan, UT, U.S.A.), penicillin/streptomycin and 2 μM α-thioglycerol (GIBCO) (Butterfield *et al*., 1988).

Mediator release experiments

For stimulation of IL-6 production, 8–14-week-old hCBMCs were washed once in each Dulbecco's phosphate-buffered saline (DPBS) and human tyrode buffer and resuspended in fresh medium containing recombinant human stem cell factor (rhSCF) (100 ng ml^{-1}) alone, which was included in the stimulation medium in all experiments for optimal mast cell viability. In anti-IgE stimulation experiments, hCBMCs were resuspended (10^6 cells ml^{-1}) and passively sensitized by incubation with human myeloma IgE (2 μg ml^{-1} 10^6 cells;

Table 1 Signal transduction pathways involved in IL-6 production from mast cells[a]

Target	*Inhibitor*[b]	*HMC-1 (IL-1, 10 ng ml^{-1})*	*hCBMCs (IL-1, 50 ng ml^{-1}) Inhibition (% total)*	*MC*[c] *(Anti-IgE) (%)*
p38 MAP kinase	SB203580 (10 μM)	56.7±2.1	52.8±10.0	0[d]
PKC	Calphostin C (0.5 μM)	80.7±6.5	95.8±1.7	
PKC α, β, μ	Gö6976 (0.5 μM)	78.3±4.5	77.1±11.2	100[e]

[a]Percent inhibition of IL-6 release during 6 h stimulation in hCBMCs or 18 h stimulation in HMC-1 cells.
[b]SB203580, Calphostin C, Gö6976 were added 30 min before IL-1.
[c]MC = mast cells; data from various sources indicated with references.
[d]hCBMCs (this study).
[e]rat peritoneal MCs (Leal-Berumen *et al*., 1994).

Chemicon) for 48–72 h in IL-6-free medium at 37°C. Cells were then washed, resuspended in medium containing rhSCF alone and distributed to 96-well 'U bottom' microtiter assay plates (1×10^5 cells 200 μl^{-1}) in duplicate or triplicate for stimulation with IL-1 (50 ng ml^{-1}, Chemicon) or anti-IgE (5–10 μg ml^{-1}; DAKO, Carpinteria, CA, U.S.A.) at 37°C in 5% CO_2 for 6 h. HMC-1 were also distributed to 96-well microtiter assay plates (2×10^5 cells 200 μl^{-1}) in duplicate or triplicate and stimulated in complete culture medium. Optimal concentrations were determined from previous dose–response and time-course experiments (Kandere-Grzybowska *et al*., 2003). IL-6 was determined in cell-free supernatants with commercial ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's directions (the sensitivity of the assay was 3 pg ml^{-1}).

For the inhibitor studies, HMC-1 or hCBMCs were stimulated in 96-well plates (2×10^5 cells 200 μl^{-1} well^{-1}) in medium containing 0.5% FBS with IL-1 (10 ng ml^{-1}) for 18 h. In order to avoid loss of cell viability, hCBMCs (1×10^5 cells 200 μl^{-1} well^{-1}) were stimulated with IL-1 (50 ng ml^{-1}) or anti-IgE (10 μg ml^{-1}) for 6 h in culture medium containing 10% FBS and SCF (but not IL-6). Inhibitors (SB203580, Calphostin C or quercetin) were added 30 min prior to stimulation; these conditions and the concentrations of the inhibitors were selected from published studies in other cell types and were tested and found to be optimal for the present studies.

Cell lysates and ELISA for phosphorylated MAP kinases

For measurements of MAP kinase phosphorylation, HMC-1 were washed two times in DPBS, resuspended in medium containing 0.5% FBS, distributed to 48-well plates (2×10^5 cells 200 μl^{-1}) in each well, and preincubated for 3 h (37°C, 5% CO_2) to reduce background induction. In order to maintain optimal cell viability, hCBMCs (2×10^5 cells 200 μl^{-1}) were stimulated in culture medium containing 10% FBS and rhSCF, but not IL-6. In preliminary studies, no difference in activation of p38 was observed in the presence or absence of either FBS or SCF (data not shown). Cells were stimulated for the indicated times with IL-1 (10 ng ml^{-1} for HMC-1 and 50 ng ml^{-1} for hCBMCs) or anti-IgE (only for hCBMCs; 10 μg ml^{-1}). Stimulation was terminated by the addition of ice cold DPBS, and cells were washed two times in DPBS. Cells were then lysed in 50 μl of cell extraction buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF and protease inhibitor cocktail from Sigma (cat # P-2714) for 30 min on ice.

Activation of MAP kinases was determined by quantifying the amounts of phosphorylated and total kinase by ELISA (Biosource International, Camarillo, CA, U.S.A.). Data in each sample was normalized against total amount of kinase and expressed as fold induction. Samples were diluted appropriately (1 : 10 or 1 : 20) to allow them to fall on the straight part of the standard curve. ELISA kits were specific for dually phosphorylated p38 MAP kinase at residues threonine 180 and tyrosine 182; ERK_1 at threonine 202 and tyrosine 204 and ERK_2 at threonine 185 and tyrosine 187; JNK_1 and JNK_2 at threonine 183 and tyrosine 185.

Immunoblotting for PKC isozymes

HMC-1 were serum starved (0.5% FBS) overnight, washed two times with PBS, and placed in six-well plates (2×10^7 cells 4 ml^{-1} well^{-1}) in fresh medium containing 0.5% FBS. IL-1 (10 ng ml^{-1}) and PMA (25 ng ml^{-1}) were added for the indicated times and stimulation was terminated by addition of ice-cold PBS. The contents of two wells were pooled (4×10^7 cells sample^{-1}) and were disrupted (4×10 s) with a Polytron homogenizer (Kinematica Inc., Cincinnati, OH, U.S.A.) in 500 μl of an extraction buffer: 50 mM Tris at pH 7.4, 2 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin. Cell extracts were centrifuged ($700 \times g$, 15 min) to remove nuclei, followed by separation of cytosolic and membrane fractions by centrifugation ($43{,}000 \times g$, 1 h). The pellet (now the membrane fraction) was washed once more with extraction buffer followed by centrifugation ($43{,}000 \times g$, 1 h). The membrane fraction was further homogenized in 500 μl extraction buffer, containing in addition 1% Triton-X. The protein concentration was determined in all samples with the BioRad protein assay. Cytosolic and membrane fractions were aliquoted and stored at −80°C. For the detection of phosphorylated forms of PKC isozymes (p-PKC), overnight serum starved (0.5% FBS) HMC-1 (4×10^6 cells 4 ml^{-1}) were stimulated with IL-1 (10 ng ml^{-1}) or PMA (25 ng ml^{-1}) for the indicated times. Cells were lysed in 200 μl SDS lysis buffer (62.5 mM Tris-HCl of pH 6.8, 2% SDS, 19% glycerol, 50 mM DTT, 0.01% bromphenol blue). Proteins (20 μg for the two fractions from 20 μl of cell lysates ~equivalent to 4×10^5 cells) were loaded on 10% Tris-HCl precast mini gels (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The separated proteins were then transferred to polyvinylidene diflouride membrane (Bio-Rad Laboratories). Immunoreactive proteins were detected by isozyme-specific antibodies at the following dilutions: PKC-α at 1 : 2000; p-PKC-μ, p-PKC-ζ, p-PKC-θ and actin at 1 : 1000; PKC-β at 1 : 250. Secondary antibody, either goat anti-mouse IgG or goat anti-rabbit IgG, was used at 1 : 2000 dilution. Blots were developed with the chemiluminescence detection system and imaged with Kodak Digital Science 1D Image Station (Eastman Kodak Company, Rochester, NY, U.S.A.).

Expression and analysis of results

The results are presented as mean ± s.e.m. of 3–7 experiments performed in duplicate or triplicate. Results were compared to control using the nonparametric Mann–Whitney *U*-test. Comparisons, among different conditions where appropriate, were made using ANOVA. Significance was set at $P < 0.05$.

Results

Selective activation of p38 MAP kinase by IL-1

In view of potential cell type specificity in IL-1 signaling, we determined the amount of total and phosphorylated forms of p38, $ERK_{1/2}$ and $JNK_{1/2}$ at the indicated time intervals, selected by preliminary studies based on the best time points shown for FcεRI (Kimata *et al*., 2000) after stimulation with IL-1 or anti-IgE of hCBMCs. Phosphorylation only of p38 was enhanced about three-fold above baseline ($n = 5$) by

stimulation with either IL-1 or anti-IgE (Figure 1a). In contrast, anti-IgE, but not IL-1, stimulated ($n=8$) the phosphorylation of $ERK_{1/2}$ about two-fold and $JNK_{1/2}$ about 15-fold in hCBMCs (Figure 1b and c). Phosphorylation of $ERK_{1/2}$ was not increased above baseline ($n=4$) by addition of IL-1, while phosphorylation of $JNK_{1/2}$ was under the detection limit in the presence or absence of IL-1. The fact that there was phosphorylation of p38 and $ERK_{1/2}$ in the absence of any stimulation suggests constitutive activation of these protein kinases in normal human mast cells. Similar results were obtained in HMC-1 cells (results not shown).

In view of the unique stimulation of p38 by IL-1, we further investigated the time course of p38 phosphorylation. Phosphorylation of p38 in hCBMCs started immediately following addition of IL-1 and peaked (about five-fold) at 15–30 min and declined close to basal levels by 2 h (Figure 2a); stimulation by anti-IgE gave a similar time course, but with peak induction that varied 3–5-fold (results not shown). The kinetics of p38 phosphorylation in response to IL-1 in hCBMCs were similar to HMC-1 cells with a peak of nine-fold at 15 min (Figure 2b). Lack of ERK and JNK phosphorylation in response to IL-1 was verified at 3, 15, 30 and 60 min in both cell types (data not shown). From these data, we conclude that p38 is the only MAP kinase activated selectively in human mast cells in response to IL-1.

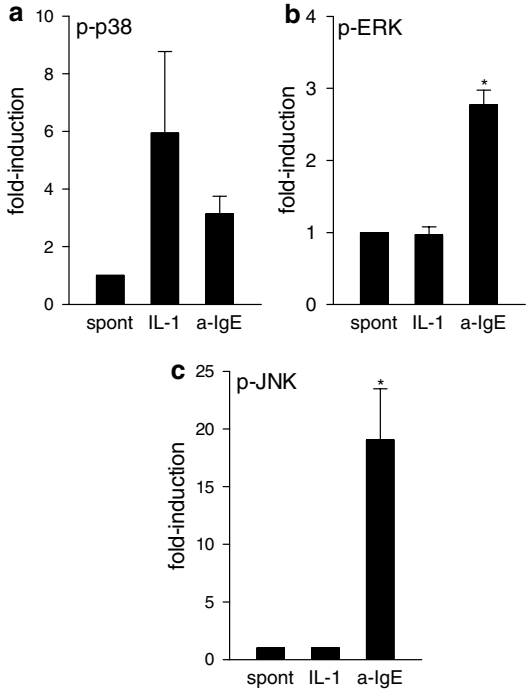

Figure 1 IL-1 selectively stimulates phosphorylation of p38 MAP kinase in hCBMCs. The phosphorylation status of MAP kinases was determined in unstimulated and IL-1α (50 ng ml^{-1})- or anti-IgE (10 μg ml^{-1})-treated hCBMCs by using ELISA. Amounts of phosphorylated and total MAPKs were determined; data were normalized and expressed as fold-induction. Cells were stimulated for 3 min for p38 (a; $n=5$), 5 min for ERK (b; $n=8$) and 15 min for JNK (c; $n=4$) assay, conditions for optimal anti-IgE activation of MAPKs. Data is mean ± s.e.m. of the number of experiments shown in the parentheses above, $^*P<0.05$ for each experimental condition compared to control; spont = spontaneous; a-IgE = anti-IgE.

Inhibition of IL-1-stimulated IL-6 production by the p38 inhibitor SB203580

The specific inhibitor for p38 MAP kinase, SB203580 (Cuenda *et al*., 1995), was used (1–10 μM) to determine the role of p38 in IL-1-induced IL-6 production in human mast cells. SB203580 (3 μM) inhibited IL-1-stimulated IL-6 production in hCBMCs by 52.8 ± 10.0% ($n=4$), but there was no statistical difference between 1 and 10 μM of the inhibitor (Figure 3a). In contrast, anti-IgE-stimulated IL-6 production was not affected by SB203580 ($n=3$; Table 1). Similarly, IL-1-stimulated IL-6 in HMC-1 cells was reduced ($n=5$) by 56.7 ± 2.1% at 10 μM SB203580 (Figure 3b). These data demonstrate that the p38 MAP kinase contributes to IL-1-stimulated IL-6 production in human mast cells.

Inhibition of IL-1-stimulated IL-6 production by PKC inhibitors

To determine whether PKC is involved in IL-1-induced IL-6 production in mast cells, we used the nonspecific PKC inhibitor Calphostin C ($IC_{50}=0.05$ μM). Pretreatment of HMC-1 cells for 30 min with Calphostin C (0.01–1 μM) prior

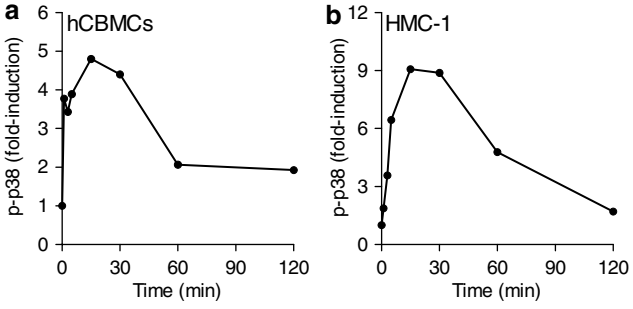

Figure 2 Kinetics of p38 phosphorylation stimulated by IL-1. The phosphorylation of p38 in IL-1-treated (a) hCBMCs (50 ng ml^{-1}) or (b) HMC-1 cells (10 ng ml^{-1}) determined by ELISA. p-p38, phosphorylated p38. Representative experiment from four experiments in duplicate is shown; data are normalized against total p38 expression.

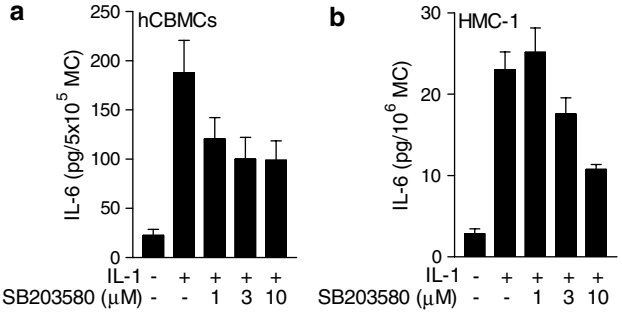

Figure 3 Inhibition of IL-1-stimulated IL-6 production by the p38 inhibitor SB203580. Cells were pretreated with SB203580 for 30 min prior to stimulation. (a) hCBMCs were stimulated for 6 h with 50 ng ml^{-1} of IL-1 ($n=5$) and (b) HMC-1 cells were stimulated for 18 h with 10 ng ml^{-1} of IL-1 ($n=5$). Data = mean ± s.e.m., $P<0.05$ for all concentrations, except for 1 μM SB253080, compared to IL-1 alone. Please note IL-6 units differ as they are listed as pg per 5×10^5 for hCBMCs, but as pg per 10^6 for HMC-1 cells due to the abundance of the latter. MC = mast cells.

to stimulation with IL-1 (10 ng ml^{-1}) inhibited ($n=4$) IL-6 production in a concentration-dependent manner (Figure 4a and b). At 0.5 μM, Calphostin C and Gö6976 inhibited IL-1-induced IL-6 production in HMC-1 cells by 80.7 $\pm$ 6.5 and 78.3 $\pm$ 4.5%, respectively. At 0.5 μM, Calphostin C also inhibited IL-6 production induced by IL-1 (50 ng ml^{-1}) in hCBMCs by 95.8 $\pm$ 1.7% (Figure 4b).

Effect of IL-1 on activation of PKC isozymes

Since Calphostin inhibited IL-1-induced IL-6 release, which had previously been shown to be calcium independent, we investigated the activation of PKC isozymes in response to IL-1 and phorbol ester (PMA) with or without the calcium ionophore A23187 (0.5 μg ml^{-1}) used as a positive control in HMC-1. These studies were performed using serum-starved (0.5% FCS) cells in order to reduce the background and increase the sensitivity of the assay. We investigated the calcium-sensitive PKC-α and β, the calcium-insensitive/PMA-sensitive PKC-ζ (the PKC isozyme most often activated by IL-1 in other cell types), as well as the calcium and PMA-insensitive PKC-θ. We investigated the translocation of PKC α, β and ζ from the cytoplasm to the membrane in HMC-1 cells by Western blotting using isozyme-specific antibodies (Figure 5). IL-1 did not stimulate translocation of PKC isozymes α, β or ζ to the plasma membrane during the observed time (0–60 min). Treatment with PMA (25 ng ml^{-1}) by itself did not have any effect but PMA (25 ng ml^{-1}) together with the calcium ionophore A23187 (0.5 μg ml^{-1}) at 30 min increased translocation of all three isozymes (Figure 5b). In contrast, PKC-θ translocated to the plasma membrane, but was not affected by PMA (not shown).

Activation of PKC isozymes was also examined by immunoblotting with antibodies specific for PKC phosphorylated isoforms. There was some basal phosphorylation of PKC-α and PKC-μ that was not further increased by IL-1, during 5–60 min stimulation; instead, PMA (25 ng ml^{-1}) stimulated its phosphorylation during the observed times (5–60 min) (Figure 5c). Activation of PKC-ζ was also evaluated by immunoblotting cell lysates with antibody to PKC-ζ phosphorylated at Thr410, which has been correlated with PKC-ζ activation. There was some baseline PKC-ζ phosphorylation in response to IL-1 while PMA stimulated its phosphorylation (Figure 5c).

IL-1 did stimulate phosphorylation of the novel PKC-θ at 15–60 min, with decrease at 120 min, as determined with immunoblotting using antibody to PKC-θ, phosphorylated at Thr538 (Figure 5c); PMA 25 ng ml^{-1} also phosphorylated this PKC isozyme at 5 min, but this effect was absent at 15 and 60 min. Equal loading was verified by immunoblotting the same membranes with antibodies to actin (Figure 5c). Pretreatment of HMC-1 with Calphostin C (1 μM) for 30 min prior to IL-1 stimulation inhibited translocation of PKC-θ ($n=2$, results not shown).

*Effect of quercetin on IL-1-induced IL-6 release and phosphorylation of PKC-*θ

Pretreatment of hCBMCs for 10 min with various amounts of quercetin prior to stimulation with IL-1 (10 ng ml^{-1}) for 30 min resulted in dose-dependent inhibition of IL-6 release that reached 79% at 100 μM (Figure 6a). Incubation of HMC-1 cells with IL-1 (10 ng ml^{-1}) for 30 min stimulated phosphorylation of PKC-θ (Thr538). However, preincubation of HMC-1 cells with quercetin (1 or 10 μM) for 15 min prior to stimulation with IL-1 (10 ng ml^{-1}) for 30 min inhibited PKC-θ phosphorylation. Equal loading was verified by immunoblotting the same membrane with antibody to actin (Figure 6b).

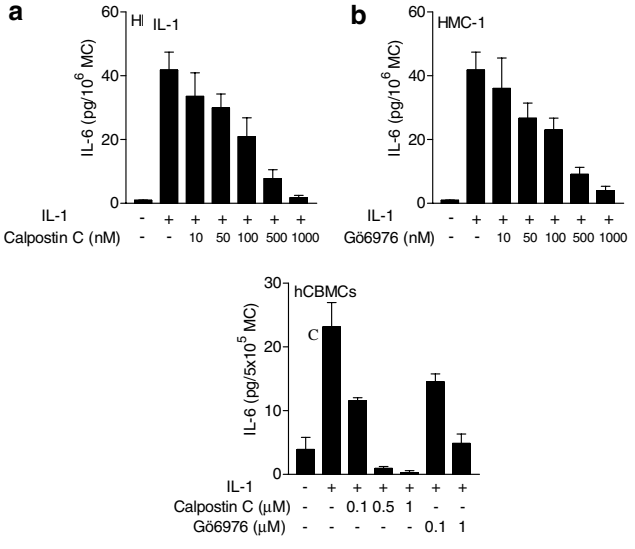

Figure 4 Inhibition of IL-1-stimulated IL-6 production by PKC inhibitors. Cells were pretreated with different concentrations of Calphostin C (a, b) for 30 min prior to stimulation with IL-1 (50 ng ml^{-1}) for 6 h for hCBMCs or 18 h for HMC-1 cells (10 ng ml^{-1}). Results are expressed as mean $\pm$ s.e.m., $n=4$, $P<0.05$ for all concentrations. MC = mast cells.

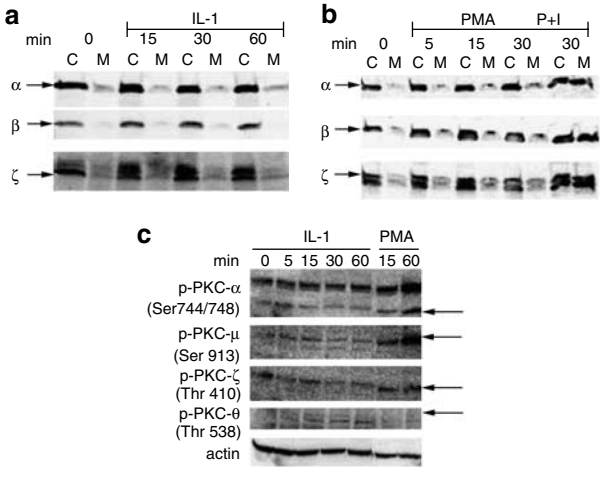

Figure 5 Effect of IL-1 on activation of PKC isozymes in HMC-1 cells. (a, b) HMC-1 cells were stimulated with IL-1 (10 ng ml^{-1}), PMA (25 ng ml^{-1}) or PMA and calcium ionophore A23187 (0.5 μg ml^{-1}); the cytosolic (C) and membrane (M) fractions were separated and immunoblotted by antibodies to PKC-α, PKC-β or PKC-ζ. (c) HMC-1 cells were stimulated with IL-1 (10 ng ml^{-1}) or PMA (25 ng ml^{-1}) for 60 min. Lysates were immunoblotted with antibodies to p-PKC-α, p-PKC-μ, p-PKC-ζ, p-PKC-θ or actin; P = phospho; P + I = PMA and ionophores. The arrows indicate the respective phospho proteins. There was only one loading control for both cytosolic and membrane fractions for all blots. Each gel is representative of three similar ones.

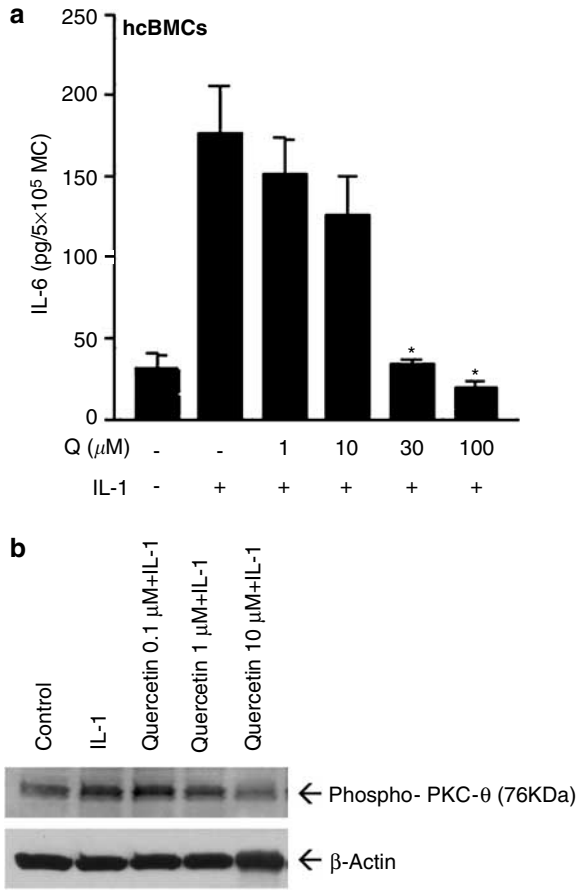

Figure 6 Effect of quercetin on IL-1-induced (a) IL-6 release ($n=4$), (b) PKC-θ phosphorylation. Incubation of HMC-1 cells with IL-1 (10 ng ml^{-1}) stimulated phosphorylation of PKC-θ (Thr538). HMC-1 cells were preincubated with quercetin (1 or 10 μM) for 15 min prior to incubation with IL-1 (10 ng ml^{-1}) for 30 min. Equal loading was verified by immunoblotting the same membrane with antibody to actin (representative gel of three similar ones). Q = quercetin.

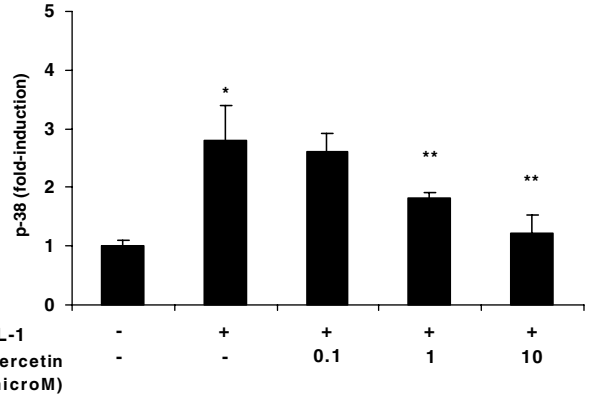

Figure 7 Effect of quercetin on IL-1-induced p38 phosphorylation. HMC-1 cells were preincubated with quercetin (0.1, 1 or 10 μM) for 15 min prior to stimulation with IL-1 (10 ng ml^{-1}) in the presence or absence of quercetin (0.1, 1 or 10 μM) for another 3 min. Cell lysates were then prepared and the phosphorylation status of p38 MAPK was determined by ELISA. Amounts of phosphorylated and total p38 was determined; data were normalized and expressed as fold-induction. Q = quercetin. Data represented as mean ± s.e.m. ($n=3$). *$P=0.05$ versus control, **$P<0.05$ versus IL-1.

Effect of quercetin on IL-1-induced p38 phosphorylation

Pretreatment of HMC-1 cells with quercetin (0.1, 1.0 or 10 μM) for 15 min prior to stimulation with IL-1 (10 ng ml^{-1}) for 30 min inhibited p38 phosphorylation in a dose-dependent manner (Figure 7).

Discussion

We have reported that IL-1 stimulates *de novo* synthesis and selective release of IL-6 without tryptase and without degranulation from human mast cells in a calcium-independent manner (Kandere-Grzybowska *et al*., 2003). In contrast to FcεRI-dependent aggregation by anti-IgE, which activates all three MAP kinases, IL-1 selectively activated only the p38 MAP kinase in HMC-1 and hCBMCs. Similar to our results, IL-1 stimulated IL-6 production in lung and synovial fibroblasts, as well as in rat cardiac myocytes, only through the p38 MAP kinase (Ridley *et al*., 1997; Miyazawa *et al*., 1998). Moreover, only the p38 MAP kinase, but not ERK or JNK, was activated by IL-1 in human neutrophils, whereas all three MAP kinases are activated by IL-1 in human monocytes, human vascular endothelial cells, human fibroblasts and human hepatoma cells (Ridley *et al*., 1997; Kumar *et al*., 1998; Suzuki *et al*., 2001). IL-1RI signaling involves activation of several tyrosine and serine/threonine protein kinases, including MAP kinases (Dinarello, 1994). To date, at least three different MAP kinases, p38, $ERK_{1/2}$ and $JNKs_{1/2}$ have been implicated in IL-1RI signaling in a cell type-specific manner (O'Neill, 2002). The MAP kinases comprise parallel signaling cascades that are known to regulate production of several cytokines by activating transcription of their target genes (Vanden Berghe *et al*., 1998).

The specific inhibitor of p38, SB203580 (Cuenda *et al*., 1995), substantially (~50%) reduced IL-1, but not anti-IgE-stimulated IL-6 production in hCBMCs, without a classic dose–response curve. These results suggest that in human mast cells, IL-1 induces some additional signals that allow mRNA induction and protein synthesis in the absence of other costimulatory signals, or the presence of some resistant p38 isoform. One possibility is that p38 stabilizes IL-6 mRNA through its AU-repeat-rich 3′-untranslated region (3′-UTR). For instance, in mouse mast cells, IL-1 did not induce transcription of IL-6 mRNA, but acted to stabilize IL-6 mRNA induced by costimulation with IL-10 and SCF (Lu-Kuo *et al*., 1996). Many cytokines, including IL-6, have AU-repeats at their 3′-UTR and p38 MAP kinase mediates the stabilization of these mRNAs in AU-rich-region-dependent manner (Miyazawa *et al*., 1998; Winzen *et al*., 1999).

The potent inhibition of IL-1-stimulated IL-6 by the PKC inhibitor Calphostin C strongly suggests the involvement of PKC. However, we did not find any evidence of activation of PKC isozymes α, β, ζ or μ in response to IL-1 in HMC-1 cells. Instead, the novel isozyme PKC-θ was uniquely activated in response to IL-1. Both classical and novel PKC isozymes have been implicated in IL-6 production due to FcεRI crosslinkage (Metzger *et al*., 2002; Blank & Rivera, 2004). PKC-β, in particular, has been linked to IgE-dependent IL-6 production in mast cells by showing that overexpression of PKC-β (but

not α, ε, δ, or η) in RBL-2H3 cells resulted in increased IL-6 mRNA levels (Chang *et al*., 1997). Moreover, *in vivo* studies implicated both PKC-β and δ isozymes since mast cells from PKC-$\beta-/-$ and PKC-$\delta-/-$ knockout mice displayed impaired FcεRI-mediated IL-6 production (Nechushtan *et al*., 2000). Long-term treatment (24 h) of rat peritoneal mast cells with PMA resulted in complete inhibition of IgE-dependent IL-6 production, implicating calcium-dependent PKC isozymes (Leal-Berumen *et al*., 1994). The atypical PKC-ζ and the novel calcium-insensitive PKC-θ have received attention due to their role in immune responses in the context of antigen receptor signaling (Isakov & Altman, 2002). PKC-$\zeta-/-$ mice have alterations in the development of secondary lymphoid organs, and induction of IL-6 mRNA is reduced in B cells (Leitges *et al*., 2001). Similar to our results, PKC-ζ did not translocate to the membrane in response to IgE and specific antigen stimulation (Ozawa *et al*., 1993a). PKC-θ was phosphorylated in response to IL-1 and by PMA, even though the timing was different. PKC-θ also translocated and was involved in activation of ERKs in response to FcεRI signaling (Liu *et al*., 2001). Moreover, PKC-θ was also shown to translocate in human T lymphocytes (Freeley *et al*., 2005) and in bone marrow-derived mouse mast cells (BMDMs) (Peng & Beaven, 2005) in response to PMA. Such apparent differences may be cell type or stimulation condition specific.

The flavonoid quercetin inhibited IL-6 secretion, as well as p38 and PKC-θ phosphorylation induced by IL-1. These results indicate that quercetin is uniquely able to inhibit this selective mast cell activation. We recently showed that quercetin could also inhibit FcεRI-induced cytokine release, as well as calcium ion elevations and PKC-θ phosphorylation (Kempuraj *et al*., 2005) in hCBMCs. Flavonoids are naturally occurring polyphenolic compounds that can inhibit a variety of enzymes (Middleton *et al*., 2000). Quercetin, the flavonoid component in Ginkgo biloba, inhibited LPS-induced TNF-α transcription by inhibiting ERK1/2 and p38 (Wadsworth *et al*., 2001). Quercetin also inhibited LPS-induced expression of TNF-α, IL-1β and IL-6 by suppressing activation of ERK and p38 MAP kinases in macrophages (Cho *et al*., 2003). Another related flavonoid, luteolin, also inhibited LPS-induced inflammatory gene expression in macrophages by reducing activation of ERK and p38 (Xagorari *et al*., 2002). Certain O-methylated catechins were also shown to inhibit FcεRI-induced histamine, cytokine and leukotriene release, as multiple protein kinases in mast cells (Maeda-Yamamoto *et al*., 2004). The ability of quercetin to inhibit allergic and nonallergic human mast cell release of IL-6 suggests that it may be useful in inflammatory conditions involving IL-6 and mast cells such as multiple sclerosis (Theoharides & Cochrane, 2004), migraines (Theoharides *et al*., 2005), inflammatory arthritis (Theoharides, 2005) and interstitial cystitis (Theoharides & Sant, 2005).

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